

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
18 March 2004 (18.03.2004)

PCT

(10) International Publication Number
WO 2004/022003 A2

- (51) International Patent Classification: **A61K**
(21) International Application Number:
PCT/US2003/028056
(22) International Filing Date:
8 September 2003 (08.09.2003)
(25) Filing Language: English
(26) Publication Language: English
(30) Priority Data:
60/319,529 6 September 2002 (06.09.2002) US

(71) Applicant (for all designated States except US): **UNIVERSITY OF SOUTH FLORIDA** [US/US]; 4202 East Fowler Avenue, FAO 126, Tampa, FL 33620-7900 (US).

(72) Inventor; and
(75) Inventor/Applicant (for US only): **MOHAPATRA, Shyam, S.** [US/US]; 18510 Country Crest Place, Tampa, FL 33647 (US).

(74) Agents: **LADWIG, Glenn, P. et al.**; Salhwanchik, Lloyd & Salhwanchik, 2421 N.W. 41st Street, Suite A-1, Gainesville, FL 32606 (US).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GH, GM, GR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NL, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **MATERIALS AND METHODS FOR TREATMENT OF ALLERGIC DISEASES**

(57) Abstract: The present invention pertains to a method for treatment of allergic diseases by administering a natriuretic hormone peptide (NHP), or a nucleic acid sequence encoding NHP, to a patient in need thereof. In another aspect, the present invention concerns an expression vector comprising a nucleic acid sequence encoding NHP. In another aspect, the present invention concerns a host cell genetically modified with a nucleic acid sequence encoding NHP. In another aspect, the present invention concerns a pharmaceutical composition comprising NHP or a nucleic acid sequence encoding NHP and a pharmaceutically acceptable carrier. In another aspect, the present invention pertains to novel fragments of atrial natriuretic peptide (ANP) exhibiting bronchodilatory and antiinflammatory activity, and isolated nucleic acid sequences encoding the fragments.

DESCRIPTIONMATERIALS AND METHODS FOR TREATMENT OF ALLERGIC DISEASES5 Cross-Reference to Related Application

This application claims the benefit of U.S. Provisional Application No. 60/319,529, filed September 6, 2002, which is hereby incorporated by reference herein in its entirety, including any figures, tables, nucleic acid sequences, amino acid sequences, or drawings.

10

Background of the Invention

Respiratory diseases, such as allergic rhinitis, asthma, and chronic obstructive pulmonary disorders (COPD) are often debilitating conditions with high prevalence, affecting more than 155 million people in the developed world. Asthma is one of the common chronic diseases and is rapidly increasing by 20% to 50% per decade, particularly in children. Currently, there are 53 million patients in the major pharmaceutical markets. Constriction of the airways is the hallmark of chronic conditions such as asthma and COPD, and inflammation is common to all respiratory diseases affecting either the upper or lower airways. Bronchodilators, which may possess limited anti-inflammatory activity, are considered the first line of therapy for asthma. Steroids are considered the gold standard as anti-inflammatory therapy, but they possess other significant adverse effects. Effective therapeutics other than steroids are under intense investigation.

A group of four peptide hormones, originating from the 126-amino acid atrial natriuretic factor (ANF) prohormone, have been known for their vasodilator activity. These four peptide hormones, consisting of amino acids 1-30, 31-67, 79-98, and 99-126 of this prohormone, have been named long acting natriuretic peptide, vessel dilator, kaliuretic peptide, and atrial natriuretic peptide (ANP), respectively for their most prominent effects (Angus R.M. *et al.*, *Clin Exp Allergy* 1994, 24:784-788). The ANP sequence, particularly the C-terminal portion, is highly conserved among species (Seidman *et al.*, *Science*, 1984, 226:1206-1209). It has been proposed to be useful for treatment of various cardiovascular, respiratory, cancerous and renal diseases (Vesely, D.L. *Cardiovascular*, 2001, 51:647-658).

The C-terminal peptide of proANF (also known by the synonym proANP), ANP is a 28-amino acid hormone secreted by the cardiac atria and lung tissue (Needleham, P. *et al.*, *N Engl J Med*, 1986, 314:828-834). ANP has vasodilator, natriuretic and diuretic properties (Needleham, P. *et al.*, *N Engl J Med*, 1986, 314:828-834). ANP infused at high concentrations reduces airway resistance in normal subjects (Hulks G. *et al.*, *Clin Sci* 1990;79:51-55) and produces a significant bronchodilator response in patients with asthma. Inhaled ANP attenuates histamine- and methacholine (MCh)-induced bronchoconstriction (Hulks, G. *et al.*, *Br. Med J*, 1992, 304:1156; Angus, R.M. *et al.*, *Clin Exp Allergy*, 1994, 24:784-788); however, the amount of ANP required for efficacy and their short half-life limits their use for long-term modulation of airway hyper-responsiveness (Hamet, P. *et al.*, *Nephrologie*, 1987, 8:7-12; Matsuse, H., *et al.*, *J Immunol*, 2000, 164:6583-6582).

The present inventor has demonstrated prolonged amelioration of symptoms associated with respiratory allergy and asthma by delivery of pDNA-encoding various natriuretic hormone peptides (NHPs), or by delivery of the peptides themselves, which exhibit bronchodilatory and/or anti-inflammatory activity.

Brief Summary of the Invention

The present invention pertains to a method for treating respiratory allergies, such as allergic rhinitis and asthma, which may be caused by allergens and exacerbated by respiratory viral infections, pollutants, and smoke.

In one embodiment, the method of the present invention comprises administering a therapeutically effective amount of a natriuretic hormone peptide (referred to herein as NHP or NHP peptide) to a patient in need of such treatment. As used herein, NHP refers to atrial natriuretic factor (ANF) hormone, or a biologically active fragment or homolog thereof.

Specifically exemplified NHPs comprise an amino acid sequence selected from the group consisting of amino acids 1-30 of ANF (also known as "long acting natriuretic peptide" and referred to herein as NHP₁₋₃₀ or SEQ ID NO:1), amino acids 31-67 of ANF (also known as "vessel dilator" and referred to herein as NHP₃₁₋₆₇ or SEQ ID NO:2), amino acids 79-98 of ANF (also known as "kaliuretic peptide" and referred to herein as NHP₇₉₋₉₈ or SEQ ID NO:3), and amino acids 99-126 of ANF (also known as "atrial natriuretic peptide" or "ANP", and referred to herein as NHP₉₉₋₁₂₆ or SEQ ID NO:4), or

biologically active fragments or homologs of any of the foregoing. Other exemplified NHPs comprise amino acids 73-102 of proANF (referred to herein as NHP₇₃₋₁₀₂ or SEQ ID NO:5), or SEQ ID NO:6, or biologically active fragment(s) or homolog(s) of the foregoing. In one embodiment, the NHP administered to the patient does not consist of

5 NHP₉₉₋₁₂₆ (SEQ ID NO:4).

In another embodiment, the method of the present invention comprises administering an effective amount of at least one nucleic acid molecule encoding an NHP to a patient in need of such treatment. The present inventor has determined that introduction of a nucleic acid molecule encoding NHP is capable of inhibiting allergen-specific IgE synthesis for the treatment of allergic disease. The gene delivery method of the present invention permits long-term expression of NHP-encoding nucleic acid sequences *in vivo*, thereby conferring bronchoprotective effect and/or anti-inflammatory effect against respiratory allergies, such as asthma. In one embodiment, a therapeutically effective amount of at least one nucleic acid molecule encoding a peptide comprising an

10 amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6 or biologically active fragments or homologs of any of the foregoing, are administered to the patient.

In another aspect, the present invention concerns an isolated peptide comprising the amino acid sequence NHP₇₃₋₁₀₂ (SEQ ID NO:5) or SEQ ID NO:6, or a biologically

20 active fragment or homolog of the foregoing. In another aspect, the present invention concerns an isolated nucleic acid molecule encoding the amino acid sequence of NHP₇₃₋₁₀₂ (SEQ ID NO:5) or encoding the amino acid sequence of SEQ ID NO:6, or a biologically active fragment or homolog thereof.

In another aspect, the present invention concerns an expression vector comprising

25 a nucleic acid sequence encoding an NHP, and a promoter sequence that is operably linked to the NHP-encoding nucleic acid sequence. In one embodiment, the expression vector is a DNA plasmid or virus. In another aspect, the present invention concerns a pharmaceutical composition comprising a nucleic acid sequence encoding an NHP, and a pharmaceutically acceptable carrier.

Brief Description of the Drawings

For a fuller understanding of the nature and objects of the invention, reference should be made to the following detailed description, taken in connection with the accompanying drawings, in which:

5 **Figure 1** shows a diagram depicting the family of natriuretic hormone peptides (NHP). Translation of the atrial natriuretic factor gene results in a pre-prohormone from which the 5' signal sequence is cleaved to yield the 126 amino acid prohormone (ANP). The prohormone is further cleaved by endopeptidases into several bioactive peptides, long-acting natriuretic peptide (LANP) (NHP₁₋₃₆; SEQ ID NO:1), vessel dilator (VD) 10 (NHP₃₁₋₆₇; SEQ ID NO:2), kaliuretic peptide (KP) (NHP₇₉₋₉₈; SEQ ID NO:3), and atrial natriuretic peptide (ANP) (NHP₉₉₋₁₂₆; SEQ ID NO:4). Urodialatin (UD), a variant of ANP is also in circulation and has been implicated in asthma. The NHP construct used in these studies encodes NHP₇₃₋₁₀₂ (SEQ ID NO:5) and SEQ ID NO:6, which are distinct from the above peptides but include a critical overlap region shared with UD and ANP.

15 **Figures 2A and 2B** show cloning and expression of NHP in human alveolar cells and effect on AHR of murine lung. Figure 2A shows the successful cloning of the peptides of ANF in the pVAX expression vector. The gel electrophoresis of excised inserts corresponding to SEQ ID NO:13 (approximately 114 bp band) and to NHP₇₃₋₁₀₂ (approximately 90 bp band) are shown. Figure 2B shows expression of NHP₇₃₋₁₀₂ in 20 human Type II alveolar epithelial cells, A549. A549 cells were either transfected with pNHP₇₃₋₁₀₂, SEQ ID NO:13 or pVAX vector control. Expression of ANP-like peptide was detected in cell supernatant and lysate from transfected cells alone but not pVAX control. *p<0.05; compared to pVAX control.

Figures 3A-3E show therapeutic effect of NHP₇₃₋₁₀₂ on asthma in mice. Figure 25 3A shows an experimental outline of immunization protocol using NHP₇₃₋₁₀₂. Figure 3B shows expression of NHP₇₃₋₁₀₂ in murine lung. Mice were administered intranasally (i.n.) either with pNHP₇₃₋₁₀₂ or pVAX as described. Three days following the last DNA administration, NHP expression was checked from lung tissue by RT-PCR. Mice receiving NHP₇₃₋₁₀₂ (lane 2) exhibited NHP expression, which was not present in control 30 mice receiving empty pVAX plasmid (lane 1). Figure 3C shows an estimation of the degree of sensitization following ova injection (Figure 3A). Mice (n=4) were injected intraperitoneally (i.p.) either with ova and alum or phosphate buffered saline (PB), and on day 21 their serum was analyzed for ova specific IgE. Mice receiving ova and alum

exhibited higher titers ($p<0.01$) of ova specific IgE than the PBS control. The experiments were repeated twice and data from a representative experiment are shown. Figure 3D-E show the measurement of AHR to increasing concentrations of methacholine following NHP gene transfer on day 26. BALB/c mice ($n=4$) were sensitized with ovalbumin by i.p. immunization ($10\text{ }\mu\text{g}/\text{mouse}$) and 14 days later were treated with $10\text{ }\mu\text{g}/\text{mouse}$ of SEQ ID NO:13 or pNHP₇₃₋₁₀₂ intranasally. The control group received the empty vector alone. Each mouse was intranasally administered three times on two days interval with $10\text{ }\mu\text{g}$ of plasmid DNA complexed with $50\text{ }\mu\text{g}$ of transfection reagent Lipofectamine (Life Technologies, Rockville, MD). Animals were challenged with the same allergen ($50\text{ }\mu\text{g}$ in PBS) three days after the last intranasal DNA delivery and 24 hours later their AHR was measured using the whole body plethysmograph (Buxco, Troy, NY). A dose-dependent decrease of methacholine response is shown in Figure 3D. Figure 3E shows the effect of treatment with SEQ ID NO:13 and pNHP₇₃₋₁₀₂ on allergen-induced airway hyper-responsiveness (AHR). The effect of treatment at the highest concentration ($50\text{mg}/\text{ml}$) of methacholine challenge is shown ($p<0.05$).

Figures 4A and 4B show the long-term effect on AHR following prophylaxis by NHP₇₃₋₁₀₂ gene transfer. Figure 4A shows schematically the protocol of sensitization, treatment and antigen challenges and measurement of AHR. Figure 4B shows measurement of Penh (%) at $50\text{mg}/\text{ml}$ of methacholine. $*p<0.05$; compared to pVAX control. The experiment was repeated twice and data from a representative experiment are shown.

Figures 5A-5C show that administration of chitosan-pNHP nanoparticles exhibit a therapeutic effect for allergen-RSV induced asthma and reversal of asthma in mice. Figure 5A shows an experimental outline of immunization protocol with allergen and RSV, treatment schedules, challenges and AHR measurements. Figure 5B shows reversal of airway hyper-reactivity as evident from % Penh measurement following treatment with chitosan + pNHP₇₃₋₁₀₂. The other treatments include chitosan + pVAX (control), chitosan + NHP₇₃₋₁₀₂, fluticasone, and fluticasone and salmeterol alone. Figure 5C shows the reduction in inflammatory cells in the lung by treatment with pNHP₇₃₋₁₀₂. Mice treated as shown in Figure 5B were subjected to bronchoalveolar lavage (BAL) following AHR. A BAL cell differential was performed and cytospun BAL cells were stained and different

cell types were quantified by three blinded investigators. The percentage of cells of macrophages, eosinophils, neutrophils and lymphocytes were determined.

Figures 6A-6C show that overexpression of NHP₇₃₋₁₀₂ leads to increased production of nitric oxide in human epithelial cells. A549 (Figure 6A) and NHBE (Figure 6B) cells were transfected with control vector or NHP₇₃₋₁₀₂. At the indicated times after transfection, aliquots of the culture medium were assayed for nitrite (the NO reaction product). Fluorescence was read at 409 nm with excitation at 365 nm using a JASCO spectrofluorometer. Data are means \pm SEM (n = 3). Figure 6C shows that NO production is due to the constitutive NOS. One aliquot of cells was incubated during the expression phase with 1 mM N^G-nitro-L-arginine methyl ester, an arginine analog that blocks eNOS production of NO (NHP+i). The enhanced NO generation was inhibited by pretreatment of the cells with N-nitro-L-arginine methyl ester, which blocks cNOS activity.

Figures 7A and 7B show that pNHP₇₃₋₁₀₂ exerts its anti-inflammatory activity in the lung by decreasing NF κ B activation in epithelial cells. A549 (Figure 7A) or NHBE (Figure 7B) cells were co-transfected with pNHP₇₃₋₁₀₂ or vector pVAX (pV) alone, NF κ B plasmid carrying the luciferase reporter gene (pNF κ B-luc reporter plasmid) (MERCURY PROFILING SYSTEM, CLONTECH), and pLacZ normalization control. NF κ B was activated 24 hr after transfection by incubating cells with 20 ng/ml phorbol myristoyl acetate (PMA) (for A549 cells) or 10ng/ml of TNF- α (for NHBE cells). Luciferase activity was detected using the DUAL LUCIFERASE REPORTER Assay kit (CLONTECH) and DYNEX MLX luminometer. Data (average of three readings \pm SEM) are expressed as fold change in luciferase activity in arbitrary units relative to vector control.

25

Brief Description of the Sequences

SEQ ID NO:1 is the amino acid sequence of human "long acting natriuretic peptide" or NHP₁₋₃₀: ¹NPMYN AVSNADLMDF KNLLDHLEEK MPLE³⁰.

30 SEQ ID NO:2 is the amino acid sequence of human "vessel dilator" or NHP₃₁₋₄₇:

³¹EVVPP QVLSEPNEEA GAALSPLPEV PPWTGEVSPA QR⁴⁷.

SEQ ID NO:3 is the amino acid sequence of human "kaliuretic peptide" or NHP⁷⁹⁻⁹⁸:
⁷⁹SSDRSAL LKSKLRALLT APR⁹⁸.

SEQ ID NO:4 is the amino acid sequence of human "atrial natriuretic peptide" (ANP) or
 5 NHP⁹⁹⁻¹²⁶:⁹⁹SLRRSSC FGGRMDRIGA QSGLGCSNFR Y¹²⁶.

SEQ ID NO:5 is the amino acid sequence of cloned mouse pNHP⁷³⁻¹⁰²:
 Gly-⁷³SPWDPSDRSALLKSKLRALLAGPRSLRR¹⁰².

10 SEQ ID NO:6 is the amino acid sequence of cloned mouse NHP fragment:
 VSNTDLMDFKNLLDHLEEKMPVEDEVMPQALSEQTE.

SEQ ID NO:7 is the amino acid sequence for the human preproANP (NCBI
 ACCESSION # NM_006172) wherein the underlined amino acids represent the signal
 15 sequence which is cleaved off to form the mature peptide:
¹MSSESTTTVS FLLLA FOLL GOTRANPMYN AVSNADLMDF KNLLDHLEEK
 MPLEDEVVPP QVLSEPNEEA GAALSPLPEV PPWTGEVSPA QRDGGALGRG
 PWDSSDRSAL LKSKLRALLT APRSLRRSSC FGGRMDRIGA QSGLGCSNFR Y¹⁵¹.

20 SEQ ID NO:8 is a forward primer for the cDNA sequence encoding mouse prepro ANP
 protein:
 5'- gac ggc aag ctt act atg ggc agc ccc tgg gac cc-3'.

SEQ ID NO:9 is a reverse primer for the cDNA sequence encoding mouse pre-proANP
 25 protein:
 5'- acc ccc ctc gag tta tta tct tcg tag gct ccg-3'.

SEQ ID NO:10 is a forward primer for the cDNA sequence encoding mouse NHP
 fragment:
 30 5'-aat cct aag ctt agt atg gtc tcc aac aca gat-3'

SEQ ID NO:11 is a reverse primer for the cDNA sequence encoding mouse NHP
 fragment:

5'- tgc gaa ctc gag tta ctc agt ctg ctc act cag ggc ctg cg-3'

SEQ ID NO:12 is the nucleotide sequence encoding cloned mouse pNHP₇₃₋₁₀₂:

atg ggc agc ccc tgg gac ccc tcc gat aga tct gcc ctc tgg aaa agc aaa ctg agg gct ctg ctc gct
5 ggc cct cgg agc cta cga aga taa

SEQ ID NO:13 is the nucleotide sequence encoding cloned mouse pNHP fragment:

atg gtg tcc aac aca gat ctg atg gat ttc aag aac ctg cta gac cac ctg gag gag aag atg cgg gta
gaa gat gag gtc atg ccc cgg cag gcc ctg agt gag cag act gag taa

10

SEQ ID NO:14 is the mRNA nucleotide sequence encoding human ANP (NCBI Accession # NM_006172:

1 tgcgcaggga cagacgtagg ccaagagagg ggaaccagag aggaaccaga ggggagagac
61 agagcagcaa gcagtgatt gctcttgac gacgccagca tgaactcctt ctccaccac
15 121 accgtgagct tctctctttt actggtcatt cagctcctag gtcagaccag agctaattcc
181 atgtacaatg ccgtgtccaa cgcagacctg atggattica agaatttgc tggacatttg
241 gaagaaanga tgcctttaga agatgaggc gtcgcccac aagtgtctag tgaagcgaat
301 gaagaaaggc gggctgtctt cagccccctt cctgaggtgc ctccctggac cggggaagtc
361 agccaccgcc agagagatgg aggtgcccct gggcggggcc cctgggactc ctctgacga
20 421 tctgcccctc taaaagcaa gctgaggggc ctgctcactg ccctcggag cctgcgaga
481 tccagctgct tcgggggcag gatggacagg attgagccc agagcggact ggcctgtaac
541 agcttcgggt actgaagata acagccagg aggacaagca gggctgggcc tagggacaga
601 tctcaagagg cctctgtccc ctggggctc tctgtcatt gtgtcatctt ttgccaatgg
661 agtgtgact atcccatcta agctgcagct tctgtcaac acttctcaca tcttatgcta
25 721 actgtagata aagtgttttg atgtgtactt cctgcctct cccaccocat gcatataatt
781 ttaagtaga acctaacctg ttactgaag tggttgaaa gtgaataaac ttacgacca
841 tggac

SEQ ID NO:15 is the human gene for atrial natriuretic factor propeptide (coding sequence
30 includes -join (570...692, 815...1141, 2235...2240); sig. peptide = 570...644; mat.
peptide = join (645...692, 815...1141, 2235...2237), (NCBI ACCESSION NO: X01471;
Greenberg, B.D. *et al.*, *Nature*, 1984, 312(5995):656-658):

1 ggaatcatt gtctgggct gctggctgcc tgcatttcc tctctccac ccttatgg

61 aggccctgac agctgagcca caaacaacc aggggagctg ggcaccagca agcgtcacc
 121 tctgtttccc cgcacggtag cagcgtcag gagaaagat cctgaggcac ggcggtgaga
 181 taaccaagga ctcttttta ctctctcac accttgaag tgggagccto ttagtcaaa
 241 tcagtaagaa tgcgcctctt gcagctgagg gtctgggggg ctgttggggc tgcccaaggc
 5 301 agagagggggc tgtgacaagc cctgcggatg ataacttaa aagggcattct cctgtggct
 361 tctcacttgg cagctttatc actgcaagtg acagaatggg gagggctctg tctctctgc
 421 gtgctggag agctgggggg ctataaaag aggcggcact gggcagctgg gagacaggga
 481 cagacgttag ccaagagagg ggaaccagag aggaaccaga gggggagagac agnagagca
 541 gcagtggtatt gctccttgac gacgccagca tgagctcctt ctccaccacc accgtgagct
 10 601 tctctctttt actggcattc cagctcctag gtcagaccag agctaatccc atgtacaatg
 661 cctgtgtcca cgcagacctg atggattca agttagggcc aggaagagcg gtgccagtctg
 721 gggccagggg gctttctgat gctgtgcta ctctcttga ttctctcaa gtcagtgaag
 781 ttatccctt tccctgtatt ttcttttct aaagaatttg ctggaccatt tgaagaaaa
 841 gatgcttta gaaatgagg tctgtccccc acaagtcttc agtgaagcca atgaagaagc
 15 901 gggggctgct ctacgcccc tccctgaggt gcttccctgg accggggag tcagccagc
 961 ccagagagat gagggtgccc tggggcgggg cccctggagc tctctgac galetgcct
 1021 cctaaaaagc aagctgaggc cgtctgtcac tgcctctgg agcctgcgga gatccagctg
 1081 ctctgggggc aggatggaca ggaatggag ccagagcgga ctgggctgta acagttccg
 1141 ggtaaagga actggggatg gaaatggat gggatggaca ctactggag acaccttag
 20 1201 caggnaagg accaatgcag aagctcattc ctctcaagt ttctcccca acaccagag
 1261 tgcccatagg gtgfcaggac atgccaatc ttgtcctag ctatgtctgt gagaatatgc
 1321 ttaaaaaaaa aagggggggg gctgggcagc gtgctcagc ctgtaatcc agcatttgg
 1381 gaggccaggc agcggatcat gagtcaaga gatcaagact atcctggcca acatgtgaa
 1441 accccagctc tactaaaaat acaaaaatta gctgggtgtg tggcggcac ctgtactctc
 25 1501 agctacttgg gagctgagg caggagaatc actgaaccc agggaggcaga ggttcagtg
 1561 agcagagatc acggcactgc agtccagcct aggtgataga gcgagactgt ctcaaaaaaa
 1621 aaaaaaaaag gccaggcgcg gtggctcagc cctgtaatcc cagcgtttg ggaggccaag
 1681 ggggttgat cagcaggtca ggaatggag acctcctgg ctaacacgtt gaaacccgt
 1741 ctctactaaa aatacaaaaa attagccagg cgtgggtgca ggcgcctgta agtcttagct
 30 1801 actccggagg ctgaggcagg agaatggct gaacccgga ggcggagctt cagctgagca
 1861 gagatggcac cactgcactc cagccgggc gacagagcaa gactccgtct caaaaaaaa
 1921 aaaaaaaa gcaactgcca ctagcactgg gaattaaaa tattcataga gccaaagtat
 1981 cttagctagg ctgatiagca gtcatattc ctccccagaa ttgcaagatc ctgaagggt

2041 taagtgaat ttactctgat gagtaacttg cttataaatt catgaagctc agagggtcat
 2101 caggctgggg tgggggcccgg tgggaagcag gtgtgcagta atcaagtica gaggatgggc
 2161 acactcatac atgaagctga ctttccagg acagccaggt caccaagcca gatatgtctg
 2221 tgttctcttt gcagtaactga agataacagc caggggaggac aagcagggtct gggccclagg
 5 2281 acagactgca agaggctcct gtccctcggg gtctctgctg catttgtctc atcttgttc
 2341 catggagttg tgcataccc atctaagctg cagcttctg tcaacacttc tcacatctta
 2401 tgctaactgt agataaagtg gtttgatggt gacttctcgc cctctccacc cccatgcatt
 2461 aaattttaag gtagaacctc acctgttact gaaagtgggt tgaagtga taaacttcag
 2521 caccatggac agagacaaa tgcctgcgtt ggtgtgcttt ctttctctt gggaaagaaa
 10 2581 ttc

SEQ ID NO:16 is the amino acid sequence for the mouse preproANP peptide

1 mgsfslitlg flvflawlpq higanpvysa vsntdlmdfks nldhleeckm pvedevmppy
 61 alseqteeag aalssalpevp pwtgevnpl rdgsalgrsp wdpsdrsal kskllallg
 15 121 prslrrscf ggnidrigaq sglgcnfry r

SEQ ID NO: 17 is the genetic sequence for the mouse preproANP peptide wherein the coding sequence starts at nucleic acid molecule position 81 and ends at nucleic acid molecule position 539.

20 1 caaaagctga gaggagaga gaaagaaacc agagtgggca gagacagcaa acatcagatc
 61 gtgcccgcac ccaagccagc atgggctcct tctccatcac cctgggcttc ttctctgtct
 121 tggccttttg gcttccagcg catattggag caaatctctgt gtacagtgcg gtgtccaaca
 181 cagatctgat gatttcaag aacctgctag accacctgga ggaagaagat ccggtagaag
 241 atgaggtcat gcccccgcag gccctgagtg agcagactga ggaagcaggg gccgcactta
 25 301 gctccctccc cgaagtgcct ccttgagctg gggaggtcaa cccacctctg agagacggca
 361 gtgtcttagg gcgcagcccc tgggaccctc ccatagatc tgcctcttg aaaaacaaac
 421 tgaaggctct gctcgttgc cctcggagcc tacgaagatc cagctgtctc ggggtagga
 481 ttgacaggat tggagccag agtggaactg gctgcaacag cttccggtac cgaagataac
 541 agccaaaggag gaaaaggcag tcgattctgc ttgacagat cgcataaagat cctnagccct
 30 601 tgttgttgtgt caccgacgtt gtgcacattg ccactgtggc gtggtgaaca cctctctgga
 661 gctgcggctt cctgcctca tctatcacga tcgatgttaa atgtatgta gtggtctagt
 721 ggggtcttgc ctctccact ctgcatatta aggtatgatc tcacctttt cagaagcag
 781 ttggaaaaaa aaaaaagaa taaacttcag caccaaggac agacgccgag gccctgatgt

841 gcttctttgg cttctgccct cagttctttg ctcctccc

Detailed Disclosure

The present invention pertains to a method for treating allergen-induced airway reactivity by administering a natriuretic hormone peptide (NHP), or a nucleic acid sequence encoding an NHP, to a patient in need thereof, thereby ameliorating airway hyper-reactivity and/or airway inflammation, which are characteristic of respiratory allergic disease, such as allergic rhinitis and asthma.

In specific embodiments, the peptides used in the subject invention comprise at least one amino acid sequence selected from the group consisting of NHP₁₋₃₀, NHP₃₁₋₆₇, NHP₇₉₋₉₈, and NHP₇₃₋₁₀₂, (SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:5, respectively), SEQ ID NO:6, or a biologically active fragment or homolog thereof. In some embodiments, a combination of NHP or NHP-encoding nucleic acid sequences is utilized. In one embodiment, the peptide utilized does not consist of the amino acid sequence of NHP₉₉₋₁₂₆ (SEQ ID NO:4). In other embodiments, the peptides used in the subject invention comprise at least one amino acid sequence selected from the group consisting of SEQ ID NO:7, and SEQ ID NO:16, or biologically active fragments or homologs of any of the foregoing.

According to the gene therapy method of the present invention, the NHP-encoding nucleic acid sequence is preferably administered to the airways of the patient, *e.g.*, nose, sinus, throat and lung, for example, as nose drops, by nebulization, vaporization, or other methods known in the art. More preferably, the nucleic acid sequence encoding NHP is administered to the patient orally or intranasally, or otherwise intratracheally. For example, the nucleic acid sequence can be inhaled by the patient through the oral or intranasal routes, or injected directly into tracheal or bronchial tissue.

In specific embodiments, the nucleic acid sequences used in the subject invention encode at least one amino acid sequence selected from the group consisting of NHP₁₋₃₀, NHP₃₁₋₆₇, NHP₇₉₋₉₈, NHP₉₉₋₁₂₆, and NHP₇₃₋₁₀₂, (SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, respectively), SEQ ID NO:6, or a biologically active fragment or homolog of any of the foregoing. In other embodiments, the nucleic acid sequences used in the subject invention comprise at least one nucleotide sequence selected from the group consisting of SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14,

SEQ ID NO:15, and SEQ ID NO:17, or a biologically active fragment or homolog of any of the foregoing.

Preferably, the nucleic acid sequence encoding the NHP is administered with a nucleic acid sequence that is operatively linked with the NHP-encoding nucleic acid sequence and operates as a regulatory sequence. For example, the regulatory sequence can be a promoter sequence that controls transcription and drives expression of the NHP-encoding nucleic acid sequence at the desired site, such as at, or adjacent to, the patient's respiratory epithelial cells. The promoter can be a constitutive or inducible promoter to allow selective transcription. The promoter can be a vertebrate or viral promoter. Optionally, enhancers may be used to obtain desired transcription levels. An enhancer is generally any non-translated nucleic acid sequence that works contiguously with the coding sequence (in *cis*) to change the basal transcription level dictated by the promoter.

The NHP-encoding nucleic acid sequences used in the methods, expression vectors, and pharmaceutical compositions of the present invention are preferably isolated. According to the present invention, an isolated nucleic acid molecule or nucleic acid sequence, is a nucleic acid molecule or sequence that has been removed from its natural milieu. As such, "isolated" does not necessarily reflect the extent to which the nucleic acid molecule has been purified. An isolated nucleic acid molecule or sequence useful in the present composition can include DNA, RNA, or any derivatives of either DNA or RNA. An isolated nucleic acid molecule or sequence can be double stranded (*i.e.*, containing both a coding strand and a complementary strand) or single stranded.

A nucleic acid molecule can be isolated from a natural source, or it can be produced using recombinant DNA technology (*e.g.*, polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis. Nucleic acid molecules can be generated or modified using a variety of techniques including, but not limited to, classic mutagenesis techniques and recombinant DNA techniques, such as site-directed mutagenesis, chemical treatment of a nucleic acid molecule to induce mutations, restriction enzyme cleavage of a nucleic acid fragment, ligation of nucleic acid fragments, polymerase chain reaction (PCR) amplification and/or mutagenesis of selected regions of a nucleic acid sequence, synthesis of oligonucleotide mixtures and ligation of mixture groups to "build" a mixture of nucleic acid molecules, and combinations thereof.

Although the phrase "nucleic acid molecule" primarily refers to the physical nucleic acid molecule and the phrase "nucleic acid sequence" primarily refers to the

sequence of nucleotides on the nucleic acid molecule, the two phrases can be used interchangeably. As used herein, a "coding" nucleic acid sequence refers to a nucleic acid sequence that encodes at least a portion of a peptide or protein (*e.g.*, a portion of an open reading frame), and can more particularly refer to a nucleic acid sequence encoding a peptide or protein which, when operatively linked to a transcription control sequence (*e.g.*, a promoter sequence), can express the peptide or protein.

The term "operably-linked" is used herein to refer to an arrangement of flanking sequences wherein the flanking sequences so described are configured or assembled so as to perform their usual function. Thus, a flanking sequence operably-linked to a coding sequence may be capable of effecting the replication, transcription and/or translation of the coding sequence. For example, a coding sequence is operably-linked to a promoter when the promoter is capable of directing transcription of that coding sequence. A flanking sequence need not be contiguous with the coding sequence, so long as it functions correctly. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence, and the promoter sequence can still be considered "operably-linked" to the coding sequence. Each nucleotide sequence coding for NHP will typically have its own operably-linked promoter sequence.

The nucleotide sequences encoding NHP used in the subject invention include "homologous" or "modified" nucleotide sequences. Modified nucleic acid sequences will be understood to mean any nucleotide sequence obtained by mutagenesis according to techniques well known to persons skilled in the art, and exhibiting modifications in relation to the normal sequences. For example, mutations in the regulatory and/or promoter sequences for the expression of a polypeptide that result in a modification of the level of expression of a polypeptide according to the invention provide for a "modified nucleotide sequence". Likewise, substitutions, deletions, or additions of nucleic acid to the polynucleotides of the invention provide for "homologous" or "modified" nucleotide sequences. In various embodiments, "homologous" or "modified" nucleic acid sequences have substantially the same biological or serological activity as the native (naturally occurring) natriuretic peptide. A "homologous" or "modified" nucleotide sequence will also be understood to mean a splice variant of the polynucleotides of the instant invention or any nucleotide sequence encoding a "modified polypeptide" as defined below.

A homologous nucleotide sequence, for the purposes of the present invention, encompasses a nucleotide sequence having a percentage identity with the bases of the nucleotide sequences of between at least (or at least about) 20.00% to 99.99% (inclusive). The aforementioned range of percent identity is to be taken as including, and providing
5 written description and support for, any fractional percentage, in intervals of 0.01%, between 20.00% and 99.99%. These percentages are purely statistical and differences between two nucleic acid sequences can be distributed randomly and over the entire sequence length.

In various embodiments, homologous sequences exhibiting a percentage identity
10 with the bases of the nucleotide sequences of the present invention can have 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent identity with the polynucleotide sequences of the instant
15 invention. Homologous nucleotide and amino acid sequences include mammalian homologs of the human NHP sequences.

The NHP homologs include peptides containing, as a primary amino acid sequence, all or part of an exemplified NHP polypeptide sequence. The NHP homologs thus include NHP polypeptides having conservative substitutions, *i.e.*, altered sequences
20 in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a peptide which is biologically active. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. In one aspect of the present invention, conservative substitutions for an amino acid within
25 the sequence may be selected from other members of the class to which the amino acid belongs (see Table 1). Conservative substitutions also include substitutions by amino acids having chemically modified side chains which do not eliminate the biological activity of the resulting NHP homolog.

Table 1.

Class of Amino Acid	Examples of Amino Acids
---------------------	-------------------------

Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

Both protein and nucleic acid sequence homologies may be evaluated using any of the variety of sequence comparison algorithms and programs known in the art. Such algorithms and programs include, but are by no means limited to, TBLASTN, BLASTP, FASTA, TFasta, and CLUSTALW (Pearson and Lipman *Proc. Natl. Acad. Sci. USA*, 1988, 85(8):2444-2448; Altschul *et al. J. Mol. Biol.*, 1990, 215(3):403-410; Thompson *et al. Nucleic Acids Res.*, 1994, 22(2):4673-4680; Higgins *et al. Methods Enzymol.*, 1996, 266:383-402; Altschul *et al. J. Mol. Biol.*, 1990, 215(3):403-410; Altschul *et al. Nature Genetics*, 1993, 3:266-272).

Identity and similarity of related nucleic acid molecules and polypeptides can be readily calculated by known methods. Such methods include, but are not limited to, those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; York (1988); Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; York (1993); Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Jersey (1994); Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Press (1987); Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; York (1991); and Carillo *et al.*, SIAM J. Applied Math., 48:1073 (1988).

The methods, pharmaceutical compositions, and vectors of the present invention can utilize biologically active fragments of nucleic acid sequences encoding the 126-amino acid atrial natriuretic factor (ANF) prohormone, such as nucleic acid sequences encoding NHP₁₋₃₀, NHP₃₁₋₆₇, NHP₇₉₋₉₈, NHP₉₉₋₁₂₆, and NHP₇₃₋₁₀₂, (SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, respectively), SEQ ID NO:6, and including biologically active fragments of the nucleic acid sequences encoding SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6.

Representative fragments of the nucleotide sequences according to the invention will be understood to mean any polynucleotide fragment having at least 8 or 9 consecutive nucleotides, preferably at least 12 consecutive nucleotides, and still more preferably at least 15 or at least 20 consecutive nucleotides of the sequence from which it is derived. The upper limit for such fragments is one nucleotide less than the total number of nucleotides found in the full-length sequence (or, in certain embodiments, of the full length open reading frame (ORF) identified herein).

In other embodiments, fragments can comprise consecutive nucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, and up to one nucleotide less than the full length ANF prohormone. In some embodiments, fragments comprise biologically active fragments of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:6.

It is also well known in the art that restriction enzymes can be used to obtain biologically active fragments of the nucleic acid sequences, such as those encoding SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6. For example, *Bal31* exonuclease can be conveniently used for time-controlled limited digestion of DNA (commonly referred to as "erase-a-base" procedures). See, for example, Maniatis *et al.* [1982] *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York; Wei *et al.* [1983] *J. Biol. Chem.* 258:13006-13512.

The methods and pharmaceutical compositions of the present invention can utilize amino acid sequences that are biologically active fragments of the 126-amino acid atrial natriuretic factor (ANF) prohormone, such as NHP₁₋₃₀, NHP₃₁₋₆₇, NHP₇₉₋₉₈, NHP₉₉₋₁₂₆, and NHP₇₃₋₁₀₂ (SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, respectively), SEQ ID NO:6, and including biologically active fragments of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6.

Representative fragments of the polypeptides according to the invention will be understood to mean any polypeptide fragment having at least 8 or 9 consecutive amino

acids, preferably at least 12 amino acids, and still more preferably at least 15 or at least 20 consecutive amino acids of the polypeptide sequence from which it is derived. The upper limit for such fragments is one amino acid less than the total number of amino acids found in the full-length sequence.

- 5 In other embodiments, fragments of the polypeptides can comprise consecutive amino acids of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, and up to one amino acid less than the full-length ANF prohormone. Fragments of polypeptides can be any portion of the full-length ANF prohormone amino acid sequence (including human or non-human mammalian homologs of the ANF prohormone) that exhibit biological activity, *e.g.*, a C-terminally or N-terminally truncated version of the ANF prohormone, or an intervening portion of the ANF prohormone. In some embodiments, fragments comprise biologically active fragments of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6.

- 20 The present invention can be practiced using other biologically equivalent forms of ANF fragments or homologs thereof as can be appreciated by the sequence comparison below. Sequence similarities between mouse and human forms of ANP are shown where areas of conservation are clearly seen.

NCBI BLAST Comparison of mouse (Query) to human (Sbjct) ANP a.a. sequences.

- 25 Query: 1 *MGSFSIT-LGFFLVLAFWLPGHIGANPVYSAVSNTDLMDFKNLLDHLLEEKMPVEDEVMP*
M SPS T + F L+LAF L G ANP+Y+AVSN DLMDFKNLLDHLLEEKMP+EDEV+PP
Sbjct: 1 *MGSFSTTTVSFILLLAFQLLGQTRANPMYNAVSADLMDFKNLLDHLLEEKMPLEDEVVPP*
- 30 Query: 60 *QALSEQTEEAGAAALSSLPVPPWTGEVNPPLRDGSALGRSPWDP*SDXXXXXXXXXXXXX
Q LSE EEAGAAALS LPEVPPWTGEV+P RDG ALGR PWD SD
Sbjct: 61 *QVLSEPNEEAGAAALSPLPEVPPWTGEVSPAQRDGGALGRGPWDSDDRSALLKSKLRALLT*
- 35 Query: 120 *GPSLRSSSCFGGRIDRIGAAQSGLGCSNFRY* 150
PSLRSSSCFGGR+DRIGAAQSGLGCSNFRY
Sbjct: 121 *APSLRSSSCFGGRMDRIGAAQSGLGCSNFRY* 151

The NHP of the invention can be prepared by well-known synthetic procedures. For example, the polypeptides can be prepared by the well-known Merrifield solid support method. See Merrifield (1963) *J. Amer. Chem. Soc.* 85:2149-2154 and Merrifield (1965) *Science* 150:178-185. This procedure, using many of the same chemical reactions and blocking groups of classical peptide synthesis, provides a growing peptide chain anchored by its carboxyl terminus to a solid support, usually cross-linked polystyrene or styrene-divinylbenzene copolymer. This method conveniently simplifies the number of procedural manipulations since removal of the excess reagents at each step is effected simply by washing of the polymer.

Alternatively, these peptides can be prepared by use of well-known molecular biology procedures. Polynucleotides, such as DNA sequences, encoding the NHP of the invention can be readily synthesized. Such polynucleotides are a further aspect of the present invention. These polynucleotides can be used to genetically engineer eukaryotic or prokaryotic cells, for example, bacteria cells, insect cells, algae cells, plant cells, mammalian cells, yeast cells or fungi cells for synthesis of the peptides of the invention.

For purposes of the present invention, the biological activity attributable to the homologs and fragments of NHP and NHP-encoding nucleic acid sequences means the capability to prevent or alleviate symptoms associated with allergic disease, such as bronchoconstriction and inflammation. This biological activity can be mediated by one or more of the following mechanisms: increased production of intracellular Ca^{++} concentration (e.g., in epithelial cells), increased production of nitric oxide (NO), and decreased activation of NFkB.

The methods of the subject invention also contemplate the administration of cells that have been genetically modified to produce NHP, or biologically active fragments thereof. Such genetically modified cells can be administered alone or in combinations with different types of cells. Thus, genetically modified cells of the invention can be co-administered with other cells, which can include genetically modified cells or non-genetically modified cells. Genetically modified cells may serve to support the survival and function of the co-administered cells, for example.

The term "genetic modification" as used herein refers to the stable or transient alteration of the genotype of a cell of the subject invention by intentional introduction of exogenous nucleic acids by any means known in the art (including for example, direct transmission of a polynucleotide sequence from a cell or virus particle, transmission of

infective virus particles, and transmission by any known polynucleotide-bearing substance) resulting in a permanent or temporary alteration of genotype. The nucleic acids may be synthetic, or naturally derived, and may contain genes, portions of genes, or other useful polynucleotides in addition to those encoding NHP. A translation initiation
5 codon can be inserted as necessary, making methionine the first amino acid in the sequence. The term "genetic modification" is not intended to include naturally occurring alterations such as that which occurs through natural viral activity, natural genetic recombination, or the like. The genetic modification may confer the ability to produce NHP, wherein the cell did not previously have the capability, or the modification may
10 increase the amount of NHP produced by the cell, *e.g.*, through increased expression.

Exogenous nucleic acids and/or vectors encoding NHP can be introduced into a cell by viral vectors (retrovirus, modified herpes virus, herpes virus, adenovirus, adeno-associated virus, lentivirus, and the like) or direct DNA transfection (lipofection, chitosan-nanoparticle mediated transfection, calcium phosphate transfection, DEAE-dextran, electroporation, and the like), microinjection, cationic lipid-mediated
15 transfection, transduction, scrape loading, ballistic introduction and infection (see, for example, Sambrook *et al.* [1989] *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).

Preferably, the exogenous nucleic acid sequence encoding NHP is operably linked
20 to a promoter sequence that permits expression of the nucleic acid sequence in a desired tissue within the patient. The promoters can be inducible or tissue specific as necessary.

The genetically modified cell may be chosen from eukaryotic or prokaryotic systems, for example bacterial cells (Gram negative or Gram positive), yeast cells, animal cells, plant cells, and/or insect cells using baculovirus vectors. In some embodiments, the
25 genetically modified cell for expression of the nucleic acid sequences encoding NHP, are human or non-human mammal cells.

According to the methods of the present invention, NHP or nucleic acid sequences encoding NHP can be administered to a patient in order to alleviate (*e.g.*, reduce or eliminate) a variety of symptoms associated with allergic diseases, in various stages of
30 pathological development, such as airway reactivity, airway inflammation, and airway remodeling. Treatment with NHP or nucleic acid sequences encoding NHP is intended to include prophylactic intervention to prevent onset of the symptoms associated with airway hyper-reactivity, airway inflammation, and airway remodeling. The nucleic acid

sequences and pharmaceutical compositions of the invention can be co-administered (concurrently or consecutively) to a patient with other therapeutic agents useful for treating airway reactivity, airway inflammation, and airway remodeling.

Expression vectors for NHP are any which are known in the art that will cause
5 expression of NHP-encoding nucleic acid sequences in mammalian cells. Suitable promoters and other regulatory sequences can be selected as is desirable for a particular application. The promoters can be inducible or tissue specific as necessary. For example the cytomegalovirus (CMV) promoter (Boshart *et al.*, *Cell*, 1985, 41:521-530) and SV40 promoter (Subramani *et al.*, *Mol. Cell. Biol.*, 1981, 1:854-864) have been found to be
10 suitable, but others can be used as well. Optionally, the NHP-encoding nucleic acid sequences used in the subject invention include a sequence encoding a signal peptide upstream of the NHP-encoding sequence, thereby permitting secretion of the NHP from a host cell. Also, various promoters may be used to limit the expression of the peptide in specific cells or tissues, such as lung cells.

15 The pharmaceutical composition of the present invention can include a liposome component. According to the present invention, a liposome comprises a lipid composition that is capable of fusing with the plasma membrane of a cell, thereby allowing the liposome to deliver a nucleic acid molecule and/or a protein composition into a cell. Some preferred liposomes of the present invention include those liposomes
20 commonly used in, for example, gene delivery methods known to those of skill in the art. Some preferred liposome delivery vehicles comprise multilamellar vesicle (MLV) lipids and extruded lipids, although the invention is not limited to such liposomes. Methods for preparation of MLV's are well known in the art. According to the present invention, "extruded lipids" are lipids which are prepared similarly to MLV lipids, but which are
25 subsequently extruded through filters of decreasing size, as described in Templeton *et al.*, *Nature Biotech.*, 1997, 15:647-652, which is incorporated herein by reference in its entirety. Small unilamellar vesicle (SUV) lipids can also be used in the composition and method of the present invention. Other preferred liposome delivery vehicles comprise liposomes having a polycationic lipid composition (*i.e.*, cationic liposomes). For
30 example, cationic liposome compositions include, but are not limited to, any cationic liposome complexed with cholesterol, and without limitation, include DOTMA and cholesterol, DOTAP and cholesterol, DOTIM and cholesterol, and DDAB and

cholesterol. Liposomes of the present invention can be any size, including from about 10 to 1000 nanometers (nm), or any size in between.

A liposome delivery vehicle of the present invention can be modified to target a particular site in a mammal, thereby targeting and making use of a nucleic acid molecule of the present invention at that site. Suitable modifications include manipulating the chemical formula of the lipid portion of the delivery vehicle. Manipulating the chemical formula of the lipid portion of the delivery vehicle can elicit the extracellular or intracellular targeting of the delivery vehicle. For example, a chemical can be added to the lipid formula of a liposome that alters the charge of the lipid bilayer of the liposome so that the liposome fuses with particular cells having particular charge characteristics. In one embodiment, other targeting mechanisms, such as targeting by addition of exogenous targeting molecules to a liposome (*i.e.*, antibodies) may not be a necessary component of the liposome of the present invention, since effective immune activation at immunologically active organs can already be provided by the composition when the route of delivery is intravenous or intraperitoneal, without the aid of additional targeting mechanisms. However, in some embodiments, a liposome can be directed to a particular target cell or tissue by using a targeting agent, such as an antibody, soluble receptor or ligand, incorporated with the liposome, to target a particular cell or tissue to which the targeting molecule can bind. Targeting liposomes are described, for example, in Ho *et al.*, *Biochemistry*, 1986, 25: 5500-6; Ho *et al.*, *J Biol Chem*, 1987a, 262: 13979-84; Ho *et al.*, *J Biol Chem*, 1987b, 262: 13973-8; and U.S. Patent No. 4,957,735 to Huang *et al.*, each of which is incorporated herein by reference in its entirety). In one embodiment, if avoidance of the efficient uptake of injected liposomes by reticuloendothelial system cells due to opsonization of liposomes by plasma proteins or other factors is desired, hydrophilic lipids, such as gangliosides (Allen *et al.*, *FEBS Lett*, 1987, 223: 42-6) or polyethylene glycol (PEG)-derived lipids (Klibanov *et al.*, *FEBS Lett*, 1990, 268: 235-7), can be incorporated into the bilayer of a conventional liposome to form the so-called sterically-stabilized or "stealth" liposomes (Woodle *et al.*, *Biochim Biophys Acta*, 1992, 1113: 171-99). Variations of such liposomes are described, for example, in U.S. Patent No. 5,705,187 to Unger *et al.*, U.S. Patent No. 5,820,873 to Choi *et al.*, U.S. Patent No. 5,817,856 to Tirosh *et al.*; U.S. Patent No. 5,686,101 to Tagawa *et al.*; U.S. Patent No. 5,043,164 to Huang *et al.*, and U.S. Patent No. 5,013,556 to Woodle *et al.*, all of which are incorporated herein by reference in their entireties).

The NHP-encoding nucleic acid sequences of the present invention can be conjugated with chitosan. For example, DNA chitosan nanospheres can be generated, as described by Roy, K. *et al.* (1999, *Nat Med* 5:387). Chitosan allows increased bioavailability of the NHP-encoding nucleic acid sequences because of protection from
5 degradation by serum nucleases in the matrix and thus has great potential as a mucosal gene delivery system. Chitosan also has many beneficial effects, including anticoagulant activity, wound-healing properties, and immunostimulatory activity, and is capable of modulating immunity of the mucosa and bronchus-associated lymphoid tissue.

Mammalian species which benefit from the disclosed methods of treatment
10 include, and are not limited to, apes, chimpanzees, orangutans, humans, monkeys; domesticated animals (*e.g.*, pets) such as dogs, cats, guinea pigs, hamsters, Vietnamese pot-bellied pigs, rabbits, and ferrets; domesticated farm animals such as cows, buffalo, bison, horses, donkey, swine, sheep, and goats; exotic animals typically found in zoos, such as bear, lions, tigers, panthers, elephants, hippopotamus, rhinoceros, giraffes,
15 antelopes, sloth, gazelles, zebras, wildebeests, prairie dogs, koala bears, kangaroo, opossums, raccoons, pandas, hyena, seals, sea lions, elephant seals, otters, porpoises, dolphins, and whales. The term "patient" is intended to include such human and non-human mammalian species. According to the method of the present invention, human or non-human mammalian NHP (or nucleic acid sequences encoding human or non-human
20 mammalian NHP) can be administered to the patient. The NHP may be naturally occurring within the patient's species or a different mammalian species. The expression vectors used in the subject invention can comprise nucleic acid sequences encoding any human or non-human mammalian NHP.

In another aspect, the present invention concerns pharmaceutical compositions
25 containing a therapeutically effective amount of NHP, or nucleic acid sequences encoding NHP, and a pharmaceutically acceptable carrier. Preferably, the NHP-encoding nucleic acid sequences are contained within an expression vector, such as plasmid DNA or virus. The pharmaceutical composition can be adapted for administration to the airways of the patient, *e.g.*, nose, sinus, throat and lung, for example, as nose drops, as nasal drops, by
30 nebulization as an inhalant, vaporization, or other methods known in the art. Administration can be continuous or at distinct intervals as can be determined by a person skilled in the art.

The pharmaceutical compositions of the subject invention can be formulated according to known methods for preparing pharmaceutically useful compositions. Furthermore, as used herein, the phrase "pharmaceutically acceptable carrier" means any of the standard pharmaceutically acceptable carriers. The pharmaceutically acceptable carrier can include diluents, adjuvants, and vehicles, as well as implant carriers, and inert, non-toxic solid or liquid fillers, diluents, or encapsulating material that does not react with the active ingredients of the invention. Examples include, but are not limited to, phosphate buffered saline, physiological saline, water, and emulsions, such as oil/water emulsions. The carrier can be a solvent or dispersing medium containing, for example, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. Formulations containing pharmaceutically acceptable carriers are described in a number of sources which are well known and readily available to those skilled in the art. For example, *Remington's Pharmaceutical Sciences* (Martin EW [1995] Easton Pennsylvania, Mack Publishing Company, 19th ed.) describes formulations that can be used in connection with the subject invention. Formulations suitable for parenteral administration include, for example, aqueous sterile injection solutions, which may contain antioxidants, buffers, bacteriostats, and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and nonaqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the condition of the sterile liquid carrier, for example, water for injections, prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powder, granules, tablets, *etc.* It should be understood that in addition to the ingredients particularly mentioned above, the formulations of the subject invention can include other agents conventional in the art having regard to the type of formulation in question.

The NHP or nucleic acid sequences encoding NHP (and pharmaceutical compositions containing them) can be administered to a patient by any route that results in prevention or alleviation of symptoms associated with allergic disease, such as bronchoconstriction and/or inflammation. For example, the NHP or NHP-encoding nucleic acid molecule can be administered parenterally, intravenously (I.V.), intramuscularly (I.M.), subcutaneously (S.C.), intradermally (I.D.), orally, intranasally,

etc. Examples of intranasal administration can be by means of a spray, drops, powder or gel and also described in US Patent No. 6,489,306, which is incorporated herein by reference in its entirety. One embodiment of the present invention is the administration of the invention as a nasal spray. Alternate embodiments include administration through
5 any oral or mucosal routes, sublingual administration and even eye drops. However, other means of drug administrations are well within the scope of the present invention.

The NHP or NHP-encoding nucleic acid molecule is administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration,
10 patient age, sex, body weight, and other factors known to medical practitioners. The pharmaceutically "effective amount" for purposes herein is thus determined by such considerations as are known in the art. For example, an effect amount of NHP-encoding nucleic acid molecule is that amount necessary to provide a therapeutically effective amount of NHP, when expressed *in vivo*. The amount of NHP or NHP-encoding nucleic
15 acid molecule must be effective to achieve improvement including but not limited to total prevention and to improved survival rate or more rapid recovery, or improvement or elimination of symptoms associated with allergen-induced airway hyper-reactivity and other indicators as are selected as appropriate measures by those skilled in the art. In accordance with the present invention, a suitable single dose size is a dose that is capable
20 of preventing or alleviating (reducing or eliminating) a symptom in a patient when administered one or more times over a suitable time period. One of skill in the art can readily determine appropriate single dose sizes for systemic administration based on the size of a mammal and the route of administration.

Standard molecular biology techniques known in the art and not specifically
25 described were generally followed as in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1989), and in Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989) and in Perbal, A Practical Guide to Molecular Cloning, John Wiley & Sons, New York (1988), and in Watson *et al.*, Recombinant DNA, Scientific American Books,
30 New York and in Birren *et al.* (eds) Genome Analysis: A Laboratory Manual Series, Vols. 1-4 Cold Spring Harbor Laboratory Press, New York (1998) and methodology as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659; and 5,272,057; and incorporated herein by reference. Polymerase chain reaction (PCR) was carried out

generally as in PCR Protocols: A Guide To Methods And Applications, Academic Press, San Diego, Calif. (1990). *In situ* (In-cell) PCR in combination with Flow Cytometry can be used for detection of cells containing specific DNA and mRNA sequences (Testoni *et al.*, *Blood*, 1996, 87:3822.)

5 The term "gene therapy", as used herein, refers to the transfer of genetic material (e.g., DNA or RNA) of interest into a host to treat or prevent a genetic or acquired disease or condition phenotype. The genetic material of interest encodes a product (e.g., a protein, polypeptide, peptide, or functional RNA) whose production *in vivo* is desired. For example, the genetic material of interest can encode a hormone, receptor, enzyme,
10 polypeptide or peptide of therapeutic value. For a review see, in general, the text "Gene Therapy" (Advances in Pharmacology 40, Academic Press, 1997).

Two basic approaches to gene therapy have evolved: (1) *ex vivo* and (2) *in vivo* gene therapy. In *ex vivo* gene therapy, cells are removed from a patient and, while being cultured, are treated *in vitro*. Generally, a functional replacement gene is introduced into
15 the cell via an appropriate gene delivery vehicle/method (transfection, transduction, homologous recombination, *etc.*) and an expression system as needed and then the modified cells are expanded in culture and returned to the host/patient. These genetically reimplanted cells have been shown to produce the transfected gene product *in situ*.

In *in vivo* gene therapy, target cells are not removed from the subject, rather the
20 gene to be transferred is introduced into the cells of the recipient organism *in situ*, that is within the recipient. Alternatively, if the host gene is defective, the gene is repaired *in situ*. These genetically altered cells have been shown to produce the transfected gene product *in situ*.

The gene expression vector is capable of delivery/transfer of heterologous nucleic
25 acid sequences into a host cell. The expression vector may include elements to control targeting, expression and transcription of the nucleic acid sequence in a cell selective manner as is known in the art. It should be noted that often the 5'UTR and/or 3'UTR of the gene may be replaced by the 5'UTR and/or 3'UTR of the expression vehicle.

The expression vector can include a promoter for controlling transcription of the
30 heterologous material and can be either a constitutive or inducible promoter to allow selective transcription. The expression vector can also include a selection gene.

Vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found generally described in Sambrook *et*

al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang *et al.*, Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega *et al.*, Gene Targeting, CRC Press, Ann Arbor, Mich. (1995),
5 Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and include, for example, stable or transient transfection, lipofection, electroporation, and infection with recombinant viral vectors.

Introduction of nucleic acids by infection offers several advantages over the other listed methods. Higher efficiency can be obtained due to their infectious nature.
10 Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the vectors to specific cell types *in vivo* or within a tissue or mixed culture of cells. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

15 A specific example of a DNA viral vector for introducing and expressing recombinant sequences is the adenovirus derived vector Adenop53TK. This vector expresses a herpes virus thymidine kinase (TK) gene for either positive or negative selection and an expression cassette for desired recombinant sequences. This vector can be used to infect cells that have an adenovirus receptor which includes most cancers of
20 epithelial origin as well as others. This vector as well as others that exhibit similar desired functions can be used to treat a mixed population of cells and can include, for example, an *in vitro* or *ex vivo* culture of cells, a tissue or a human subject.

Additional features can be added to the vector to ensure its safety and/or enhance its therapeutic efficacy. Such features include, for example, markers that can be used to
25 negatively select against cells infected with the recombinant virus. An example of such a negative selection marker is the TK gene described above that confers sensitivity to the antibiotic gancyclovir. Negative selection is therefore a means by which infection can be controlled because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the
30 viral vector or recombinant sequence, cellular transformation will not occur. Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type.

In addition, recombinant viral vectors are useful for *in vivo* expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

In another aspect, the present invention concerns an isolated peptide comprising the amino acid sequence NHP₇₃₋₁₀₂ (SEQ ID NO:5), or a biologically active fragment or homolog thereof. NHP₇₃₋₁₀₂ is amino acids 73-102 of the 151-amino acid long human atrial natriuretic factor (ANF)). In another aspect, the present invention concerns an isolated peptide comprising the amino acid sequence of SEQ ID NO:6, or a biologically active fragment or homolog thereof. SEQ ID NO:6 is a biologically active fragment of the human ANF. In another aspect, the present invention concerns an isolated nucleic acid molecule encoding the amino acid sequence of NHP₇₃₋₁₀₂ (SEQ ID NO:5), or a biologically active fragment or homolog thereof. In another aspect, the present invention concerns an isolated nucleic acid molecule (SEQ ID NO:13) encoding the amino acid sequence of SEQ ID NO:6, or a biologically active fragment or homolog thereof.

As used herein, the terms "peptide", "polypeptide", and "protein" refer to amino acid sequences of any length unless otherwise specified.

Example 1—Expression of NHP in human type-II alveolar A549 cells and murine lung

A. Materials and Methods

Animals. Six-week old female BALB/c mice from Jackson laboratory (Bar Harbor, ME) were maintained in pathogen free conditions in accordance with animal research committee regulations.

Construction of NHP expression vector. Total RNA was isolated from murine heart using Trizol reagent (LIFE TECHNOLOGY, Gaithersburg, MD) following the manufacturer's protocol. The cDNA sequence for the 151 amino acid long pre-pro hormone ANF was amplified by RT-PCR. SEQ ID NO:13 was amplified using primers

listed in SEQ ID NO:8 and SEQ ID NO:9. NHP₇₃₋₁₀₂ was amplified using primers listed in SEQ ID NO:10 and SEQ ID NO:11. A translation initiation codon was inserted in the forward primers (SEQ ID NO:8 and SEQ ID NO:10), so that the recombinant peptides had an additional amino acid, methionine, as the first amino acid apart from its known amino acid content. The PCR product was cloned in pVAX vector (INVITROGEN, Carlsbad, CA) at HindIII and XhoI sites. The cloned NHP₇₃₋₁₀₂ sequence was verified by DNA sequencing and its expression was checked in A549 human epithelial cells.

Estimation of NHP by EIA. The expression of NHP was measured by utilizing a commercial kit (SPI-BIO, France) according to the manufacturer's instructions. The kit measures the rat NHP, which is homologous to the mouse NHP used in the present study.

B. Results

NHP was amplified using PCR and the product was cloned into pVAX vector, as described. Either pNHP₇₃₋₁₀₂ or empty plasmid vector pVAX were transfected to the human type-II alveolar cells (A549). To confirm the expression of NHP, cells and supernatants were collected 48 hours post-transfection and NHP concentrations were measured by ELISA. Cells transfected with pNHP produced NHP in both supernatant and cell extracts (Figure 2A), whereas the cells transfected with pVAX did not. These results show that the NHP₇₃₋₁₀₂ peptide was expressed and secreted into the culture medium from the A549 cells.

Example 2—pNHP delivered with lipofectin intranasally attenuates airway reactivity in ovalbumin-sensitized mice

A. Materials and Methods

Animals. Six-week old female BALB/c mice from Jackson laboratory (Bar Harbor, ME) were maintained in pathogen free conditions in accordance with animal research committee regulations.

Allergen Sensitization, Intranasal Gene Transfer. BALB/c mice (n=6) were sensitized once with intraperitoneal (i.p.) injection of 10 µg ovalbumin (OVA) precipitated with 1 mg of alum on day 1. Mice were intranasally (i.n.) administered three times a day with either 25 µg/mouse pNHP₇₃₋₁₀₂ or vector control (pVAX) (10 µg of lipofectamine in PBS) on days 15, 18 and 21, as shown in Figure 3A.

RT-PCR analysis. Total RNA was isolated from murine lung and spleen tissue using Trizol reagent (LIFE TECHNOLOGY, Gaithersburg, MD) and RT-PCR was

performed utilizing ANP specific primers as described before (Kumar M. *et al.*, *Vaccine* 1999; 18:558-567). The resultant PCR products were analyzed by electrophoresis on a 1.5% agarose gel and the products visualized by staining with ethidium bromide.

Measurement of Ova specific IgE. Ova specific IgE was measured to monitor the degree of sensitization. Microtitre plates were coated overnight at 4°C with 100 µl of OVA (5 µg/ml). Sera obtained at day 21 from sensitized and non-sensitized mice (n=4) were incubated to the antigen-coated wells and bound IgE was detected with biotinylated anti-mouse IgE (02112D; Pharmingen, CA). Biotin anti-mouse IgE (02122D) reacts specifically with the mouse IgE of Igh^a and Igh^b haplotypes and does not react with other IgG isotypes. Diluted streptavidin-peroxidase conjugate was added, the bound enzyme detected with TMB, and the absorbance read at 450 nm.

Pulmonary Function. Airway hyperreactivity was measured in animals three days after the last intranasal DNA delivery following 24 hours of challenge with the ovalbumin (50 µg/mouse using a whole body plethysmograph (BUXCO, Troy, NY), as previously described (Matsuse, H. *et al.*, *J. Immunol.*, 2000, 164:6583-6592). Allergen-induced airway hyper-responsiveness (AHR) was measured on 10 days interval up to day 56. Mice were ova challenged 24 hours prior to each AHR measurement.

Statistical analysis. Pairs of groups were compared by the student's t-test. Differences between groups were considered significant at $p < 0.05$. Values for all measurements are expressed as the mean \pm SD.

B. Results

The effect of pNHP gene transfer was examined in an ovalbumin-sensitized BALB/c mouse model. RT-PCR was performed to confirm the expression of ANP in murine lung and spleen. ANP specific transcript was observed in the lung tissue of mice receiving pANP construct and not from the mice receiving the empty plasmid vector (Fig. 1B). No expression of ANP was observed from the spleen tissue (data not shown).

Ovalbumin-specific IgE was measured in the serum to determine the degree of sensitization achieved following ovalbumin injection. Mice that received ovalbumin with alum had significantly ($p < 0.01$) higher ovalbumin-specific IgE titers than the control group of mice that received PBS (Figure 3C).

AHR was measured in BALB/c mice, sensitized with ovalbumin and administered with pNHP₇₃₋₁₀₂ or control vector plasmid prior to ovalbumin challenge. The control

mice received empty vector. The experimental outline is shown in Figure 3A. Mice administered pNHP showed significantly less AHR ($p < 0.05$) than did controls to inhaled methacholine (Figure 3D). Prophylactic treatment with either plasmid (SEQ ID NO:13) or pNHP₇₃₋₁₀₂ showed a reduction in % Penh suggesting that both plasmid are capable of prophylactically attenuating AHR.

To determine the length of protection, following pNHP gene transfer mice were challenged at 10 d interval. The protection lasted for over a period of 25 days during which the AHR of pNHP treated mice was significantly lower ($P < 0.05$) than the mice receiving control plasmid (Figure 4 A and B). Although there was a decrease in the AHR on day 56, the differences between values were not significant.

Example 3—Chitosan-pNHP nanoparticles administered intranasally decrease airway hyper-reactivity and inflammation

A. Materials and Methods

Animals. Female 6 to 8 week-old wild type BALB/c mice from Jackson Laboratory (Bar Harbor, ME) were maintained in pathogen-free conditions at the University of South Florida College of Medicine vivarium. All procedures were reviewed and approved by the committees on animal research at the University of South Florida College of Medicine and VA Hospital.

Preparation of chitosan-pNHP nanoparticles. pNHP₇₃₋₁₀₂ encoding DNA was cloned in the mammalian expression vector pVAX (INVITROGEN, San Diego, CA), and complexed with chitosan, as described previously (Hulks, G. *et al.*, *Clin Sci*, 1990, 79:51-55; Angus, R.M. *et al.*, *Clin Exp Allergy*, 1994, 24:784-788). Briefly, recombinant plasmid dissolved in 25 mM Na₂SO₄ was heated for 10 min at 55°C. Chitosan (VANSON, Redmond, WA) was dissolved in 25 mM Na acetate, pH 5.4, to a final concentration of 0.02% and heated for 10 min at 55°C. After heating, chitosan and DNA were mixed, vortexed vigorously for 20-30 seconds, and stored at room temperature until use.

Reversal of established AHR. Mice were sensitized i.p. with 50 µg OVA/alum on day 1 followed by intranasal challenge with 50 µg of OVA on day 14. On days 21-23, mice were given 25 µg of pNHP/chitosan i.n. per mouse. Mice were further challenged

i.n. with OVA (50 µg/mouse) on days 27 through 29 and AHR was measured on day 30. Mice were bled and sacrificed on day 31, and spleens and lungs were removed.

Examination of bronchoalveolar lavage fluid (BALF). Mice were sacrificed and lungs were lavaged with 1 ml of PBS introduced through the trachea. The BALF was centrifuged 10 min. at 300 xg and cells were rinsed with PBS and resuspended. Aliquots of the cell suspension were applied to slides using a CYTOSPIN apparatus (SHANDON SOUTHERN), stained and examined microscopically. Cells were identified as monocytes, eosinophils, neutrophils, and lymphocytes by morphological characteristics. Two slides per mouse (n=4) were counted by three blinded investigators.

Statistical analysis. Pairs of groups were compared by the student's *t*-test. Differences between groups were considered significant at $p < 0.05$. Values for all measurements are expressed as the mean \pm SD.

B. Results

A combination of allergen exposure and respiratory syncytial virus induces chronic asthma phenotype in BAALB/c mice. To determine whether therapeutic administration of chitosan-pNHP can attenuate established asthma induced by allergen exposure and RSV infection, mice were first sensitized and challenged with OVA and then infected with RSV and subsequently given Chitosan+pNHP (CHiP) therapy, as shown in the protocol of Figure 5A. Airway hyper-reactivity (%Penh) was measured by whole body plethysmography (Figure 5B). The results show a complete reversal to the basal level of AHR in the group of mice that were treated with CHiP. To determine whether CHiP therapy decreases established pulmonary inflammation, lungs of treated and then OVA-challenged mice were lavaged and BAL cells were examined. The number of eosinophils in the BAL fluid showed a significant reduction in the CHiP-treated mice compared with the untreated control group, as shown in Figure 5C.

Example 4—pNHP induces production of nitric oxide by activating constitutive nitric oxide synthase in human in lung epithelial cells

A. Materials and Methods

Cell lines and culture conditions. The human alveolar type II epithelial cell line A549 (ATCC) was cultured at 37°C in Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum and 100 U/ml each of penicillin and streptomycin in an

atmosphere of 5 % CO₂/95 % air. Cells were subcultured weekly and used between passages 9 and 22. Experiments were repeated with primary NHBE obtained from CLONETICS (Walkersville, MD) from pooled donors. These cells were cultured in BEBM medium supplied by the vendor and supplemented with 10 % fetal bovine serum and a mix of growth factors without antibiotics. Cells were grown at 37°C in 5 % CO₂/95 % and used between passages 3 and 9.

Expression plasmids and transfection. The construction of plasmid encoding NHP₇₃₋₁₀₂ has been described previously (Kumar, M *et al.*, *J Allergy Clin Immunol.*, 2002, 110:879-82). For transfection of epithelial cells, cells at 60% confluence (log phase) were transfected 4 hr at 37°C with plasmid DNA (1 µg per 10⁶ cells) complexed with lipofectamine (GIBCOBRL Life Technologies). Complete medium was then added to the cultures and the cells were incubated at 37°C for 24 to 48 h to allow expression of the natriuretic peptides.

Assay for nitric oxide. The assay for nitric oxide (NO) is based on that of Misko *et al.* (Misko, TP *et al.*, *Anal Biochem.*, 1993, 214:11-6) and measures nitrite, the stable breakdown product of NO, which is reacted with diaminonaphthalene to produce a fluorescent compound. A549 or NHBE cells were transfected with plasmid/lipofectamine complexes as described above. At specific time points, 100 µl samples of culture medium were removed and stored at -20°C. After all samples were taken, they were cleared by centrifugation and 10 µl of a freshly prepared solution of 0.02 mg/ml diaminonaphthalene was added to each tube, shaken, and allowed to react for 10 min at room temperature. The reaction was stopped by addition of 30 µl of 0.5 M NaOH and the fluorescence of the samples was read using a quartz microcuvet (3 mm path length) in a JASCO spectrofluorometer with excitation at 365 nm and emission at 409 nm. Nitrite standards were run in the same medium as the experimental samples to generate a standard curve which was used to calibrate the readings. As a positive control, one set of wells was incubated with 1 µM calcium ionophore, A23187 (SIGMA).

B. Results

NO is a bronchodilator and Ca⁺⁺-calmodulin binding activates the constitutive form of nitric oxide synthase (cNOS) in epithelial cells (Howarth, P.H. *et al.*, *Int Arch Allergy Immunol.*, 1995, 107:228-30). To determine whether the increased intracellular Ca⁺⁺ seen in NHP₇₃₋₁₀₂-transfected cells affects nitric oxide (NO) levels, aliquots of the medium

were removed before the Ca^{++} assay and mixed with diaminonaphthalene which reacts with nitrite (from the reaction of NO and water) to produce a fluorescent compound. NO generation was significantly higher in cells expressing NHP₇₃₋₁₀₂ (Fig. 3A and B). To verify that NO production was due to the constitutive NOS, one aliquot of cells was incubated during the expression phase with 1 mM N_ω-nitro-L-arginine methyl ester, an arginine analog that blocks cNOS production of NO. The enhanced NO generation was inhibited by pretreatment of the cells with N-nitro-L-arginine methyl ester, which blocks cNOS activity (Fig.3C). Airway smooth muscle hypertrophy and hyperplasia are important determinants of airway remodeling and bronchial responsiveness in asthma. NHP₇₃₋₁₀₂ appears to act on epithelial cells to produce NO via constitutive NOS, which in turn controls bronchial hyperreactivity and proliferation of airway smooth muscle cells.

Example 5—pNHP induces anti-inflammatory response in the lung by decreasing NFκB activation of epithelial cells

15 *A. Materials and Methods*

Luciferase reporter assay for NFκB activation. A549 and NHBE cells were grown to about 60 % confluence in 12-well culture plates and transfected using LIPOFECTAMINE 2000 (INVITROGEN, Carlsbad CA). Cells were transfected with a luciferase construct under the control of an NFκB-activatable promoter (MERCURY PROFILING SYSTEM, CLONTECH, Palo Alto CA) and pLacZ as a normalization control either with or without pANP. Relative amounts of plasmid DNA and lipofectamine reagent were optimized for NHBE cells and cells were transfected for 4 h in serum-free DMEM without antibiotics at 37°C. After transfection, DMEM with 10 % FBS was added and cells were incubated for 24 to 48 h at 37°C. Cells were harvested at specific time points and lysates were assayed for luciferase activity using the DUAL LUCIFERASE Assay System (PROMEGA, Madison WI) read in an MLX microplate luminometer (DYNEX TECHNOLOGIES, Chantilly VA). Transfection efficiencies were normalized by measuring β-galactosidase activity.

Statistical analysis. Experiments were repeated a minimum of three times and data are expressed as means ± SEM. Pairs of groups were compared through the use of Student's *t* tests. Differences between groups were considered significant at $p \leq 0.05$.

B. Results

To study the potential role of NHP in regulating inflammation, A549 cells were transfected with pNHP plasmid encoding amino acids 73-102 (pNHP₇₃₋₁₀₂) and the activation of NF κ B, which controls a number of genes encoding proinflammatory molecules and is key to the inflammatory cascade and has been linked to inflammation, was examined (Ishii, Y. *et al.*, *J Anat.*, 1989, 166:85-95; Boiteau, R. *et al.*, *Am Rev Res Dis.*, 1988, 137:A484). The results showed that cells cotransfected with pNHP significantly decreased luciferase activity compared to control plasmid suggesting that pNHP is capable of inhibiting PMA-induced NF κ B activation in A549 cells and in NHBE cells, as shown in Figures 6A-6B. These results indicate that pNHP possesses potential anti-inflammatory activities, and that it may exert its bronchodilatory effect by stimulating the production of NO and its anti-inflammatory effect by deactivating NF κ B, the central molecule controlling inflammation in asthmatic airways.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

Claims

What is claimed is:

1. A method of treating allergic disease comprising administering a natriuretic hormone peptide (NHP), or a nucleic acid sequence encoding NHP and an operably-linked promoter sequence, to a patient in need thereof.
2. The method of claim 1, wherein said administering comprises administering the NHP to the patient, and wherein the NHP comprises an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:6, or a biologically active fragment or homolog of any of the foregoing.
3. The method of claim 1, wherein said administering comprises administering the nucleic acid sequence encoding NHP to the patient, and wherein the NHP comprises an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6, or a biologically active fragment or homolog of any of the foregoing.
4. The method of claim 1, wherein said administering is by a route selected from the group consisting of oral, intramuscular, parenteral, intravenous, and intranasal.
5. The method of claim 1, wherein the NHP or the nucleic acid sequence is administered with a pharmaceutically acceptable carrier.
6. The method of claim 1, wherein said administering comprises administering the nucleic acid sequence to the patient, and wherein the nucleic acid sequence is contained within an expression vector.
7. The method of claim 5, wherein the expression vector is a DNA plasmid or virus.

8. The method of claim 1, wherein said administering comprises administering the nucleic acid sequence to the patient, and wherein the nucleic acid sequence is administered with chitosan.

9. The method of claim 1, wherein the NHP or nucleic acid sequence causes a bronchodilatory effect in the patient.

10. The method of claim 1, wherein the NHP or nucleic acid sequence inhibits airway reactivity, airway inflammation, or airway remodeling in the patient.

11. The method of claim 1, wherein the patient is human.

12. The method of claim 1, wherein the patient is suffering from asthma.

13. A pharmaceutical composition comprising a natriuretic hormone peptide (NHP); or a nucleic acid sequence encoding NHP, and an operably linked promoter sequence; and a pharmaceutically acceptable carrier.

14. The pharmaceutical composition of claim 13, wherein said composition comprises said NHP, and wherein said NHP comprises an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:6, or a biologically active fragment or homolog of any of the foregoing.

15. The pharmaceutical composition of claim 13, wherein said composition comprises said nucleic acid sequence encoding NHP and said operably linked promoter sequence, and wherein said NHP comprises an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6, or a biologically active fragment or homolog of any of the foregoing.

16. The pharmaceutical composition of claim 13, wherein said composition comprises an expression vector containing said nucleic acid sequence and said operably linked promoter sequence.

17. The pharmaceutical composition of claim 16, wherein said expression vector is a DNA plasmid or virus.

18. The pharmaceutical composition of claim 13, wherein said composition is an inhalant.

19. The pharmaceutical composition of claim 13, wherein said composition is an intranasal spray, drops, gels, or powder.

20. An expression vector comprising a nucleic acid sequence encoding a natriuretic hormone peptide (NHP) and an operably linked promoter sequence.

21. The expression vector of claim 20, wherein said NHP comprises an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6, or a biologically active fragment or homolog of any of the foregoing.

22. The expression vector of claim 20, wherein said expression vector is a DNA plasmid or virus.

23. An isolated cell genetically modified with a nucleic acid sequence encoding a natriuretic hormone peptide (NHP) and an operably linked promoter.

24. The isolated cell of claim 23, wherein said NHP comprises an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6, or a biologically active fragment or homolog of any of the foregoing.

25. An isolated nucleic acid sequence encoding an amino acid sequence consisting essentially of SEQ ID NO:5 or SEQ ID NO:6, or encoding a biologically active fragment or homolog of SEQ ID NO:5 or SEQ ID NO:6.

26. An isolated peptide comprising an amino acid sequence consisting essentially of SEQ ID NO:5 or SEQ ID NO:6, or a biologically active fragment or homolog of SEQ ID NO:5 or SEQ ID NO:6.

1/8

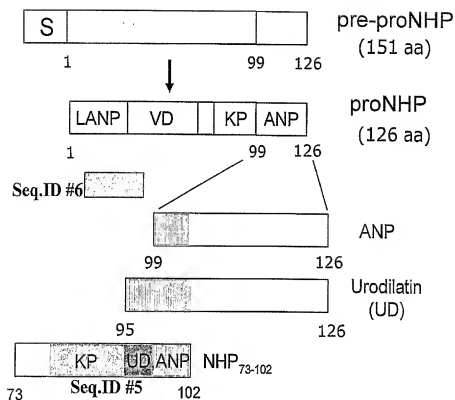


FIG. 1

2/8

FIG. 2A

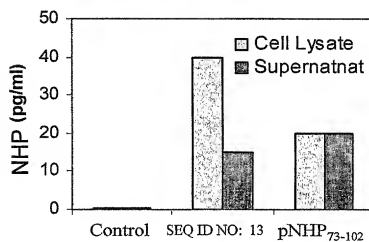
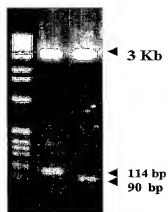


FIG. 2B

FIG. 3A

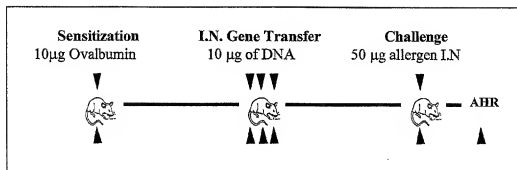


FIG. 3B

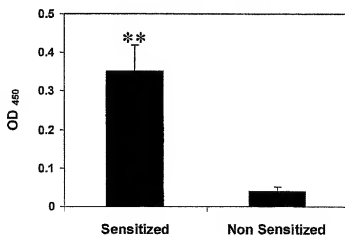
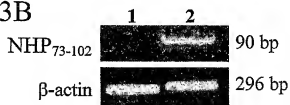


FIG. 3C

4/8

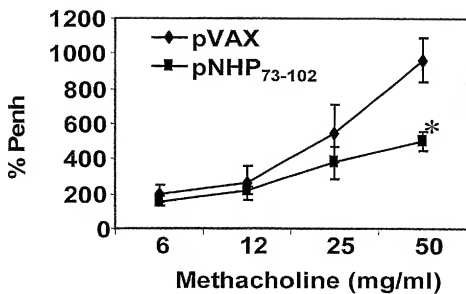


FIG. 3D

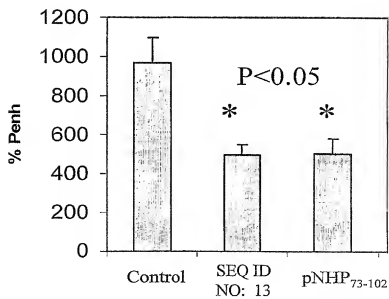


FIG. 3E

5/8

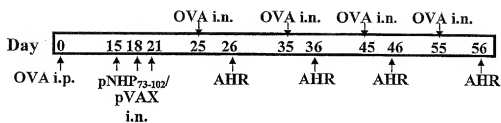


FIG. 4A

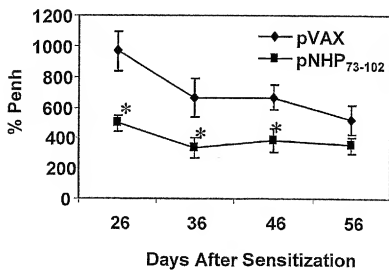


FIG. 4B

6/8

FIG. 5A

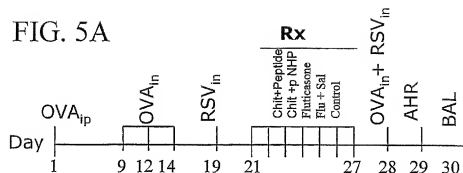


FIG. 5B

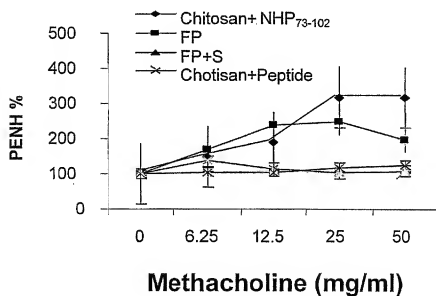


FIG. 5C

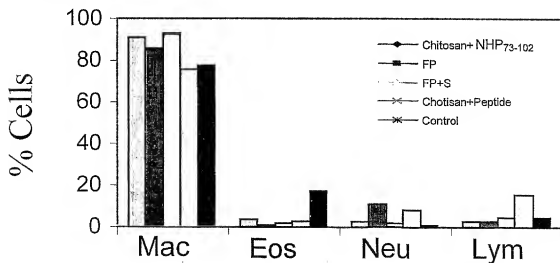


FIG. 6A

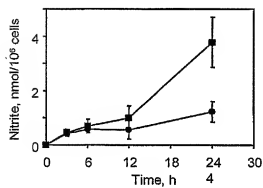


FIG. 6B

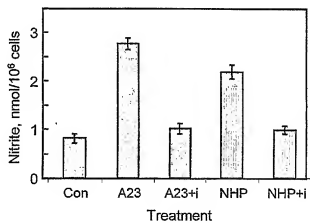
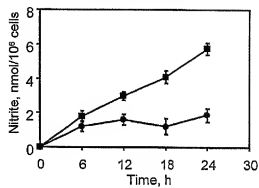


FIG. 6C

8/8

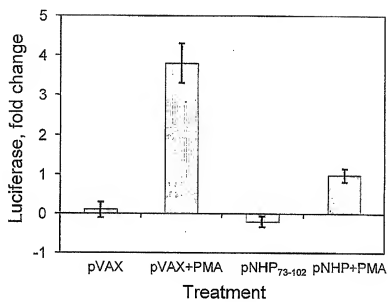


FIG. 7A

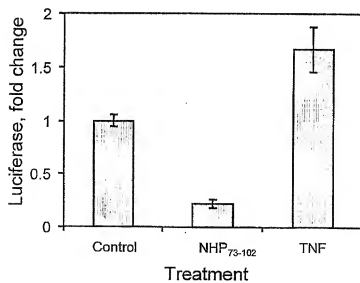


FIG. 7B

SEQUENCE LISTING

<110> University of South Florida

<120> Materials and Methods for Treatment of Allergic Diseases

<130> USP-183XC1 PCT

<150> 60/319,529

<151> 2002-09-06

<160> 17

<170> PatentIn version 3.1

<210> 1

<211> 30

<212> PRT

<213> Homo sapiens

<400> 1

Asn Pro Met Tyr Asn Ala Val Ser Asn Ala Asp Leu Met Asp Phe Lys
1 5 10 15

Asn Leu Leu Asp His Leu Glu Glu Lys Met Pro Leu Glu Asp
20 25 30

<210> 2

<211> 37

<212> PRT

<213> Homo sapiens

<400> 2

Glu Val Val Pro Pro Gln Val Leu Ser Glu Pro Asn Glu Glu Ala Gly
1 5 10 15

Ala Ala Leu Ser Pro Leu Pro Glu Val Pro Pro Trp Thr Gly Glu Val
20 25 30

Ser Pro Ala Gln Arg
35

<210> 3

<211> 20

<212> PRT

<213> Homo sapiens

<400> 3

Ser Ser Asp Arg Ser Ala Leu Leu Lys Ser Lys Leu Arg Ala Leu Leu
1 5 10 15

Thr Ala Pro Arg
20

<210> 4

<211> 28

<212> PRT

<213> Homo sapiens

<400> 4

Ser Leu Arg Arg Ser Ser Cys Phe Gly Gly Arg Met Asp Arg Ile Gly
1 5 10 15

Ala Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr
20 25

<210> 5

<211> 28

<212> PRT

<213> Mus musculus

<400> 5

Ser Pro Trp Asp Pro Ser Asp Arg Ser Ala Leu Leu Lys Ser Lys Leu
1 5 10 15

Arg Ala Leu Leu Ala Gly Pro Arg Ser Leu Arg Arg
20 25

<210> 6

<211> 37

<212> PRT

<213> Mus musculus

<400> 6

Val Ser Asn Thr Asp Leu Met Asp Phe Lys Asn Leu Leu Asp His Leu
1 5 10 15

Glu Glu Lys Met Pro Val Glu Asp Glu Val Met Pro Pro Gln Ala Leu
20 25 30

Ser Glu Gln Thr Glu
35

<210> 7

<211> 151

<212> PRT

<213> Homo sapiens

<400> 7

Met Ser Ser Phe Ser Thr Thr Thr Val Ser Phe Leu Leu Leu Leu Ala
1 5 10 15

Phe Gln Leu Leu Gly Gln Thr Arg Ala Asn Pro Met Tyr Asn Ala Val
20 25 30

Ser Asn Ala Asp Leu Met Asp Phe Lys Asn Leu Leu Asp His Leu Glu
35 40 45

Glu Lys Met Pro Leu Glu Asp Glu Val Val Pro Pro Gln Val Leu Ser

<213> Mus musculus

<400> 10
aatcctaagc ttagtatggt gtccaacaca gat 33

<210> 11

<211> 41

<212> DNA

<213> Mus musculus

<400> 11
tgcgaaactcg agttactcag tctgctcact cagggcctgc g 41

<210> 12

<211> 93

<212> DNA

<213> Mus musculus

<400> 12
atggggcagcc cctgggaccc ctccgataga tctgccctct tgaaaagcaa actgagggct 60
ctgctcgctg gcctcggag cctacgaaga taa 93

<210> 13

<211> 117

<212> DNA

<213> Mus musculus

<400> 13
atgggtgtcca acacagatct gatggatttc aagaacctgc tagaccacct ggaggagaag 60
atgcogtag aagatgaggt catgcccccg caggccctga gtgagcagac tgagtaa 117

<210> 14

<211> 845

<212> DNA

<213> Homo sapiens

<400> 14

tgggcgaggga cagacgtagg ccaagagagg ggaaccagag aggaaccaga ggggagagac	60
agagcagcaa gcagtggatt gctccttgac gacgccagca tgagctcctt ctccaccacc	120
accgtgagct tctcctcttt actggcattc cagctcctag gtcagaccag agctaattcc	180
atgtacaatg cagtgtccaa cgcagacctg atggatttca agaatttgct ggaccatttg	240
gaagaaaaga tgcctttaga agatgaggtc gtgccccac aagtgtcag tgagccgaat	300
gaagaagcgg gggctgctct cagcccccct cctgagggtgc ctccctggag cggggaagt	360
agcccgaccc agagagatgg aggtgccctc gggcggggcc cctgggactc ctctgacga	420
tctgcctctc taaaaagcaa gctgaggggc ctgctcactg cccctcggag cctcgggaga	480
tccagctgct tcgggggcag gatggacagg attggagccc agagcggact gggctgtaac	540
agcttccggt actgaagata acagccaggg aggacaagca gggctgggccc tagggacaga	600
ctgcaagagg ctctctgtcc ctgggggtctc tgcctgattt gtgtcatctt gtgcccag	660
agttgtgac atccactcta agctgcagct tctgttcaac acttctcaca tcttatgta	720
actgtagata aagtgggttg atgggtgaatt cctcgcctct cccaccccat gcattaaatt	780
ttaaggtaga acctcacctg ttactgaaag tgggtttgaa gtgaataaac ttacgaccca	840
tgga.c	845

<210> 15

<211> 2583

<212> DNA

<213> Homo sapiens

<400> 15

ggatccattt gtctcgggct gctgggtgcc tggcatttcc tctctccac ccttatattg	60
aggccctgac agctgagcca caaacaacc aggggagctg ggcaccagca agcgtcaccc	120
tctgtttccc cgcacggtac cagcgtcgag gagaagaat cctgaggcac ggcggtgaga	180
caaccaagga ctctttttta ctctctcac acctttgaag tgggagcctc ttgagtcaaa	240
tcagtaagaa tgcggctctt gcagctgagg gctcgggggg ctgtgggggc tgcacaaggc	300
agagaggggc tgtgacaagc cctcgggatg ataactttaa aagggcattc cctcgtggct	360
tctcacttgg cagctttatc actgcaagtg acagaatggg gagggtctg tctctcctgc	420

gtgcttgagg agctgggggg ctataaaaag aggcggcact gggcagctgg gagacagggg 480

cagacgtagg ccaagagagg ggaaccagag aggaaccaga ggggagagac agagcagcaa 540

gcagtgagatt gctccttgac gacgccagca tgagctcctt ctccaccacc accgtgagct 600

tcctcctttt actggcattc cagctcctag gtcagaccag agctaatacc atgtacaatg 660

cogtgtccaa cgcagacotg atggatttca aggtaggggc aggaagagcg gtgcagctcg 720

gggocagggg gctttctgat gctgtgtca ctctcttga ttctctcaa gtocagtggg 780

tttatccctt tcctctgtatt ttcttttctt aaagaatttg ctggaccatt tggagaagaa 840

gatgccctta gaagatgagg tcgtgcccc acaagtgtc agtgagocga atgaagaagc 900

ggggcgctgt ctcagccccc tcctgaggt gcctccctgg accggggag tcagccagc 960

ccagagagat ggaggtgcc tcgggcgggg cccctgggac tcctctgac gatctgcc 1020

cttaaaaagc aagctgaggc cgtgtgtcac tgcctctcg agcctgogga gatccagctg 1080

cttcgggggc aggatggaca ggaattggagc ccagagocga ctgggtgtga acagcttcg 1140

ggtaagaagg actggggatg gaaatgggat gggatggaca ctactgggag acaccttcag 1200

caggaaaggg accaatgcag aagctcattc cctctcaagt ttctgcccc acacccagag 1260

tgcccatagg gtgtcaggac atgccatcta ttgtccttag ctagtctgct gagaaaaatgc 1320

ttaaaaaaa aagggggggg gctgggcacg gtgtcacgc ctgtaatacc agcactttgg 1380

gaggccagcg agcgatcat gaggtaaga gatcaagact atcctggcca acatggtgaa 1440

acccagctc tactaaaaat acaaaaatta gctgggtgtg tggcgggcac ctgtactctc 1500

agctacttgg gaggtgagg caggagaatc acttgaacc aggaaggcaga ggttgcatg 1560

agcagagatc accccactgc agtccagcct aggtgataga gcgagactgt ctcaaaaaa 1620

aaaaaaaag gccaggcgcg gtggctcacg cctgtaatc cagcgcttgg ggaggccaa 1680

gogggtggat cagaggtca ggagatggag accatcctgg ctaaacaggt gaaacccct 1740

ctctactaaa aatacaaaaa attagccagg cgtgggtggca ggcgctgtga agtctagct 1800

actcggagg ctgaggcagg agaattggct gaaccogggg ggcggagctt gcagtggca 1860

gagatggcac caactgcact cagcctgggc gacagagcaa gactccgtct caaaaaaaa 1920

aaaaaaaaa gcaactgcac ctagcactgg gaaataaaa tattcataga gccaaagtat 1980

ctttgcattg ctgattagca gttcatatto ctcccagaa ttgcaagatc ctgaagggt 2040

taagtgaat ttactctgat gagtaacttg ottatcaatt catgaagctc agagggctcat 2100

caggctgggg tggggggcgg tgggaagcag gtggtcagta atcaagttca gaggatgggc 2160

acactcatac atgaagctga ctttccagg acagccaggt caccagcca gatatgtctg 2220

tgttctctt gcagtactga agataacagc cagggaaggc aagcagggtc gggcctaggg 2280

```

acagactgca agaggtcctt gtccctggg gtctctgctg catttggtgc atcttggtgc 2340
catggagttg tgatcatccc atctaagctg cagcttctcg tcaacacttc tcacatctta 2400
tgctaactgt agataaagtg gtttgatggt gacttctctg cctctccac cccatgcatt 2460
aaattttaag gtagaacctc acctgttact gaaagtgtt tgaagtgaa taaacttcag 2520
caccatggac agaagacaaa tgcctgcgtt ggtgtgcttt cttctctctt gggaagagaa 2580
ttc 2583

```

<210> 16

<211> 152

<212> PRT

<213> Mus musculus

<400> 16

```

Met Gly Ser Phe Ser Ile Thr Leu Gly Phe Phe Leu Val Leu Ala Phe
1           5           10           15

```

```

Trp Leu Pro Gly His Ile Gly Ala Asn Pro Val Tyr Ser Ala Val Ser
          20           25           30

```

```

Asn Thr Asp Leu Met Asp Phe Lys Asn Leu Leu Asp His Leu Glu Glu
          35           40           45

```

```

Lys Met Pro Val Glu Asp Glu Val Met Pro Pro Gln Ala Leu Ser Glu
50           55           60

```

```

Gln Thr Glu Glu Ala Gly Ala Ala Leu Ser Ser Leu Pro Glu Val Pro
65           70           75           80

```

```

Pro Trp Thr Gly Glu Val Asn Pro Pro Leu Arg Asp Gly Ser Ala Leu
          85           90           95

```

```

Gly Arg Ser Pro Trp Asp Pro Ser Asp Arg Ser Ala Leu Leu Lys Ser
          100          105          110

```

```

Lys Leu Arg Ala Leu Leu Ala Gly Pro Arg Ser Leu Arg Arg Ser Ser
          115          120          125

```

```

Cys Phe Gly Gly Arg Ile Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly
          130          135          140

```

Cys Asn Ser Phe Arg Tyr Arg Arg
145 150

<210> 17

<211> 878

<212> DNA

<213> Mus musculus

```

<400> 17
caaaagctga gagagagaga gaaagaaacc agagtgggca gagacagcaa acatcagatc      60
gtgccccgac ccacgccagc atgggctcct tctccatcac cctgggcttc ttctctcgtct      120
tggccttttg gcttccaggc catattggag caaatcctgt gtacagtgcg gtgtccaaca      180
cagatctgat ggatttcaag aacctgctag accacctgga ggaagaagatg ccggtagaag      240
atgagggtcat gcccccgcag gccttgagtg agcagactga ggaagcaggg gccgcactta      300
gtccccctcc cgaggcgctt ccctggactg gggagggtcaa cccacctctg agagacggca      360
gtgctctagg gcgcagcccc tgggacccct ccgatatagc tgcctctctg aaaagcaaac      420
tgagggctct gctcgctggc cctcggagcc tacgaagatc cagctgcttc gggggtagga      480
ttgacaggat tggagcccag agtggactag gctgcaacag cttcoggtac cgaagataac      540
agccaaggag gaaaaggcag tcgattctgc ttgagcagat cgcaaaagat cctaagccct      600
tgtggtgtgt cgcgcagctt ggtcacattg ccactgtggc gtggtgaaca cctccttgga      660
gtgcggctt cctgccttca tctatcacga tcgatgttaa atgtagatga gtggtctagt      720
ggggtcttgc ctctccact ctgcataatta aggtagatcc tcaccctttt cagaagaagag      780
ttggaaaaaa aaaaaaagaa taaacttcag caccaaggac agacgccagc gccctgatgt      840
gcttcttttg ctctgcctt cagttctttg ctctcccc      878

```